

AD _____

Award Number: DAMD17-98-1-8244

TITLE: Characterization of Tubulin Isoforms in Breast Cancer

PRINCIPAL INVESTIGATOR: Asok Banerjee, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health
Science Center at San Antonio
San Antonio, Texas 78284-7828

REPORT DATE: May 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010504 183

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2000	3. REPORT TYPE AND DATES COVERED Annual (1 May 99 - 30 Apr 00)	
4. TITLE AND SUBTITLE Characterization of Tubulin Isoforms in Breast Cancer			5. FUNDING NUMBERS DAMD17-98-1-8244	
6. AUTHOR(S) Asok Banerjee, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas Health Science Center at San Antonio San Antonio, Texas 78284-7828 E-MAIL: BANERJEE@UTHSCSA.EDU			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Tubulin, the dimeric protein of microtubules, exists as various isoforms in different tissues and species. Tubulin is the target protein for various antitumor drugs such as paclitaxel, vinblastine and vincristine, which are routinely used for cancer chemotherapy. Previous studies from this laboratory have shown that certain tubulin isoform exhibit preferential interaction with antitumor drugs. Thus, the isoform composition of a tissue may affect the antimitotic properties of any drug. Here, breast cancer cells were tested for the presence of different tubulin isoforms by immunoblotting and RT-PCR analysis. Breast cancer cells were made resistant to antitumor drugs paclitaxel and podophyllotoxin and the tubulin isoform content was studied. The results show that paclitaxel-resistant MCF-7 cells had 2-3 fold increase in β_{II} and β_{III} expression than that of the wild type. On the other hand, podophyllotoxin-resistant MDA-MB-231 cells had an increased expression of β_{VI} than that of the wild type cells. A full length cDNA for β_{III} was prepared. This cDNA will be used to construct a plasmid for the overexpression of β_{III} tubulin in the breast cancer cells. The cells will be tested for the sensitivity to antitumor drugs. These results will be very important for proper selection as well as design of novel drugs for breast cancer.				
14. SUBJECT TERMS Breast Cancer, tubulin, maytansine, paclitaxel, podophyllotoxin, vinblastine, colchicine, MCF-7, MDA-MB-231				15. NUMBER OF PAGES 13
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ ____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


 6/7/00
PI - Signature Date

TABLE OF CONTENTS

FRONT COVER.....	1
STANDARD FORM (SF) 298, REPORT DOCUMENTATION PAGE.....	2
Foreword	3
TABLE OF CONTENTS.....	4
INTRODUCTION.....	6
BODY.....	7
KEY RESEARCH ACCOMPLISHMENTS.....	10
REPORTABLE OUTCOMES AND CONCLUSIONS	
APPENDICES	

Approved Statement of Work

TASK 1: MONTHS 1 - 6:

- To grow the breast cancer cells
- To isolate tubulin from the breast cancer cells by paclitaxel-induced assembly
- To quantitate each β -tubulin isoform by SDS-PAGE and immunoblotting

TASK 2: MONTHS 7 - 12:

- To grow the breast cancer cells
- To isolate tubulin from the breast cancer cells by paclitaxel-induced assembly
- To study the post-translational modifications of tubulin

TASK 3: MONTHS 13 - 20:

- To grow the breast cancer cells in the presence of antitumor drugs
- To determine the IC_{50} values for different antitumor drugs

TASK 4: MONTHS 21 - 36:

- To study the interaction of anti-tumor drugs with purified tubulin isoforms from bovine brain
- To study the drug effects on the assembly and dynamics of microtubules

Introduction

Tubulin, the $\alpha\beta$ dimeric protein of microtubules, is the target of various antitumor drugs such as paclitaxel, vinblastine, and vincristine. Both α - and β -tubulin occur as different isoforms which are expressed in a tissue-specific manner. Our earlier results have demonstrated that the antimitotic drugs colchicine and its analogs exhibit differential interaction with different β -tubulin isoforms. The primary goal of this project is to study the role played by individual tubulin isoforms in the drug sensitivity of breast cancer cells. There are about 5-6 α -tubulin and as many as 6-7 β -tubulin forms in human system. Efforts will be initiated to study the β -tubulin isoforms in the breast cancer cells. Furthermore, to test whether isoform composition can affect drug sensitivity, full length cDNA specific for different tubulin isoforms will be prepared and then will be inserted into breast cancer cells. The

Report Body

Preparation of drug-resistant Breast Cancer Cells:

It has been reported that certain tubulin isoforms get expressed when cancer cells get resistant to anticancer drugs. To study the tubulin isoforms efforts were initiated to prepare breast cancer cells resistant to antimetabolic drugs. The cell lines were prepared by initially growing breast cancer cell lines MCF-7 and MDA-MB-231 in the presence of 1 nM of colchicine, podophyllotoxin, vinblastine or paclitaxel. Verapamil was kept in the growth medium to exclude multidrug-resistant cells. The drug concentration was gradually increased by 1.5 times. After 3-4 months of selection, two drug-resistant lines **MCF-7/PTX20** (resistant to paclitaxel) and **MDA-MB-231/POD60** (resistant to podophyllotoxin) were obtained.

Immunoblot analysis of β -tubulin isoforms in drug-resistant breast cancer cells:

The drug-resistant breast cancer cells were grown in suitable medium in T-150 culture flasks to confluency. The cells were trypsinized and harvested after washing twice in sterile PBS. The cell pellets were homogenised in PBS using 1 ml glass homogeniser and homogenate was centrifuged at 20,000 rpm in a sorvall centrifuge for 1 h. The cell extract was mixed with equal volume of 2X Laemmli sample buffer, boiled for 5 min, and was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal antibodies to β_{II} , β_{III} , and β_{IV} .

As shown in figure 1, the paclitaxel-resistant MCF-7 cells contain much higher amounts of β_{II} and β_{III} as compared to the drug-sensitive wild type cells. The amount of β_{IV} was increased marginally in the resistant cells. On the other hand, podophyllotoxin-resistant cells exhibited a decrease in the content of all three isoforms β_{II} , β_{III} , and β_{IV} . Since no antibody was available, it was not possible to see the status of the other β -tubulin isoforms by immunoblotting.

RT-PCR studies:

To study the expression of different β -tubulin isoforms, total RNA was isolated from breast cancer cells. The RNA was subjected to RT-PCR amplification and was visualized in agarose gels after staining with ethidium bromide.

β -tubulin-specific primers used for the polymerase chain reaction

<u>CLASS</u>	<u>POSITION</u>	<u>SEQUENCE</u>
I (HM40)	5'-UTR	Forward: 5'- ACCTCGCTGCTCCAGCCTCT-3' Reverse: 5'- CCGGCCTGGATGTGCACGAT-3'
II (H β 9)	Coding	Forward: 5'- CGCATCTCCGAGCAGTTCAC-3' Reverse: 5'- TCGCCCTCCTCCTCCTCGA-3'
III (H β 4)	3'-UTR	Forward: 5'- CTGCTCGCAGCTGGAGTGAG-3' Reverse: 5'- CATAAATACTGCAGGAGGGC-3'
IV a (H β 5)	5'-UTR	Forward: 5'- TCTCCGCCGCATCTTCCACC-3' Reverse: 5'- CCGGCCTGGATGTGCACGAT-3'
IV b (H β 2)	5'-UTR	Forward: 5'- GAGCTTGCCAGCCTCGTTCT-3' Reverse: 5'- CCGATCTGGTTGCCGCACTG-3'
VI (H β 1)	5'-UTR	Forward: 5'- ACAGTGTGTTGGCTCACACC-3' Reverse: 5'- CCGATCTGGTTGCCGCACTG-3'
Human GAPDH		Forward: 5' GTT CGA CAG TCA GCC GCA TCT 3' Reverse: 5' GGC ATG GAC TGT GGT CAT GAG 3'

The expression of different β -tubulin isoforms was studied in MCF-7 cells as well as in paclitaxel resistant MCF-7/PTX20 cells by RT PCR amplification of total RNA. As shown in fig. 2, the expression of β_I , β_{II} , β_{III} and β_{VI} is increased significantly. On the other hand β_{IVa} and β_{IVb} is decreased.

At this point it is not clear why the level of some of the isoforms gets elevated while that of others decrease. It may be possible that cells can identify those isoforms that have the lowest interaction with the drug, and specifically overexpress those isoforms, while the isoforms that have the highest affinity for the drug get lower expression. In order to test this hypothesis, individual α - and β -tubulin

Preparation of full length cDNA for β_{III} tubulin

To overexpress individual tubulin isoforms it will be necessary to make full length cDNA specific for individual tubulin isoforms. By using primers specific for β_{III} tubulin we have prepared full length 1350 bp cDNA from total RNA isolated from MCF-7 cells by RT-PCR. The product shows a single 1350 bp band in 1.5% agarose gel. We are planning to construct a plasmid for the overexpression of this full length cDNA in breast cancer cells.

Key Research Accomplishments

- We have studied the expression of tubulin isoforms in breast cancer cell lines by immunoblotting and RT-PCR analysis.
- Paclitaxel resistant MCF-7/PTX20 cells express increased amounts of β_{II} , β_{III} but not β_{IV} .
- Podophyllotoxin resistant MDA-MB-231/POD60 cells express lower amounts of β_{II} , β_{III} , and β_{IV} .
- RT-PCR analysis of the total RNA from paclitaxel resistant cells show the increased expression of β_{II} , and β_{III} , while an inhibition β_{IVb} is observed.
- We have prepared a full length cDNA for β_{III} from MCF-7 cells.

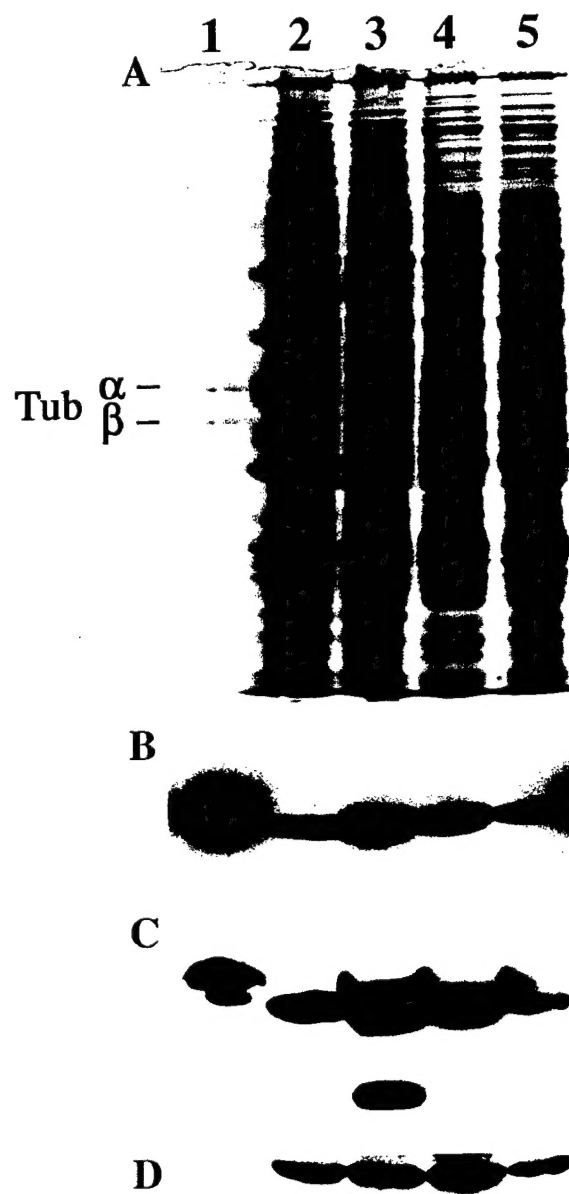
Figure Legends:

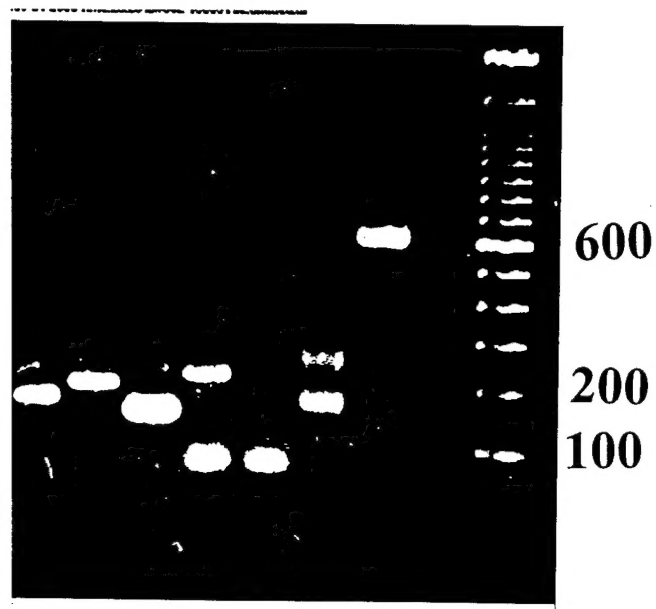
Figure 1. Immunoblotting analysis of the β -tubulin isoforms in the cell extracts from drug-resistant Breast cancer cells

The cell extracts were boiled with Laemmli sample buffer for 5 min and were subjected to SDS PAGE on 7.5 % polyacrylamide gel (Panel A). Three identical gels were transblotted onto nitrocellulose membranes for immunoblotting. The blots after the transfer were blocked with 5% milk and 0.1% BSA for 2h, incubated with antibodies specific for the β -tubulin isoforms for 1h and washed to remove the free antibody. The blots were subsequently incubated with horseradish peroxidase-coupled secondary antibody for 1 h. After washing off the secondary antibody, the blots were developed with an enhanced chemiluminescent HRP-substrate and exposed on Kodak XOMat X-ray films. The samples are: Lane 1, PC-tubulin from brain, lane 2: MCF-7 extract, lane 3, MCF-7/PTX20, lane 4, MDA-MB-231 extract, lane 5, MDA-MB-231/POD60 extract. Loading in lanes 2-5 was 50 μ g. Panel A: Gel; Panel B-D, Immunoblots. B, Anti- β_{II} ; C, Anti- β_{III} , and D, Anti- β_{IV} . The region for α - and β - tubulin is indicated on the gel.

Figure 2. RT-PCR analysis of the β -tubulin isoforms in drug resistant breast cancer cells

Total RNA (1mg) from MCF-7 and MCF-7/PTX20 cells were first incubated with DNase I at room temperature for 10 min and then reverse transcribed with 2 units AMV reverse transcriptase for one hour in the presence of deoxyribonucleotides and oligo (dT) primers. An aliquot of each sample was amplified in the presence of Taq DNA polymerase for 32 cycles using primers specific for β -tubulin isoforms. The samples after amplification were loaded on a 1.5% agarose gel in tris-EDTA buffer. Ethidium bromide (0.5 μ g/ml) was included in the running buffer for immediate visualization of the DNA bands. Upper panel: Wild type MCF-7 cells; Lower panel: Paclitaxel resistant MCF-7/PTX20 cells. Individual β -tubulin isoforms are indicated by roman numerals, G stands for GAPDH.





I II III IV a b VI G S

